

Available online at www.sciencedirect.com







www.elsevier.com/locate/vaccine

Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge *

Patti J. Miller, Daniel J. King, Claudio L. Afonso, David L. Suarez*

Southeast Poultry Research Laboratory, Agricultural Research Services, United States Department of Agriculture, 934 College Station Road, Athens, GA 30605, USA

> Received 17 May 2007; received in revised form 3 July 2007; accepted 12 July 2007 Available online 3 August 2007

Abstract

Strains of Newcastle disease virus (NDV) can be separated into genotypes based on genome differences even though they are antigenically considered to be of a single serotype. It is widely recognized that an efficacious Newcastle disease (ND) vaccine made with any NDV does induce protection against morbidity and mortality from a virulent NDV challenge. However, those ND vaccines do not protect vaccinates from infection and viral shed from such a challenge. Vaccines prepared from ND viruses corresponding to five different genotypes were compared to determine if the phylogenetic distance between vaccine and challenge strain influences the protection induced and the amount of challenge virus shed. Six groups of 4-week-old specific pathogen-free Leghorn chickens were given oil-adjuvanted vaccines prepared from one of five different inactivated ND viruses including strains B1, Ulster, CA02, Pigeon84, Alaska196, or an allantoic fluid control. Three weeks post-vaccination, serum was analyzed for antibody content using a hemagglutination inhibition assay against each of the vaccine antigens and a commercial NDV ELISA. After challenge with virulent CA02, the birds were examined daily for morbidity and mortality and were monitored at selected intervals for virus shedding. All vaccines except for the control induced greater than 90% protection to clinical disease and mortality. The vaccine homologous with the challenge virus reduced oral shedding significantly more than the heterologous vaccines. NDV vaccines formulated to be phylogenetically closer to potential outbreak viruses may provide better ND control by reducing virus transmission from infected birds.

Published by Elsevier Ltd.

Keywords: Vaccine; Exotic Newcastle disease; Immunity

1. Introduction

Newcastle disease virus (NDV), also known as avian Paramyxovirus type-1 virus, is a member of the genus *Avulavirus* [1] in the Paramyxoviridae family. It is a single stranded, non-segmented, enveloped RNA virus with negative polarity [2]. NDV is composed of six genes and their

corresponding six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and the RNA polymerase (L). RNA editing of the P protein produces two additional proteins, V and W. The HN and F are glycoproteins that allow binding and fusion of the virus to the host cells to initiate a NDV infection. Antibodies to HN and F are neutralizing and represent the primary protective component induced by Newcastle Disease (ND) vaccines [3].

Antigenic [4] and genetic diversity [5] are recognized within the APMV-1 serotype. At least six distinct lineages of NDV have been identified based on restriction enzyme analysis and nucleotide sequence of the fusion protein gene [5,6]. Another classification system using full-length sequence to

 $^{^{\}dot{\pi}}$ Proprietary or brand names used are necessary to report factually on available data. However, the USDA neither guarantees nor warrants the standard of the product, and the use of names by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

^{*} Corresponding author. Tel.: +1 706 546 3479; fax: +1 706 546 3161. E-mail address: david.suarez@ars.usda.gov (D.L. Suarez).

relate the viruses isolated over time has been reviewed by Lomniczi and coworkers [7] and shows two major divisions represented by Class I and Class II, with Class II being further divided into at least eight genotypes. This paper will refer to the second classification system when discussing the ND viruses used. The amino acid diversity across NDV sequences available on GenBank® for both the HN and the F genes displays on average a 10% difference between the genotypes of Class II and a 15% difference between Class I and Class II viruses. Amino acid diversity among strains may have been the basis of the report in 1951 that certain NDV strains were antigenically superior to others when used to formulate a killed vaccine [8].

Historically, NDV isolates have been divided into three groups used to describe their virulence in poultry: lentogen (low virulence), mesogen (moderate virulence) and velogen (high virulence) [2]. Select lentogenic strains are universally used as live vaccines in the commercial poultry industry. Experimental infections of specific pathogen-free (SPF) chickens with these lentogenic vaccine strains cause little to no clinical disease. When these viruses are used in the field they can cause decreased productivity in commercial chickens by inducing a mild respiratory disease, particularly when the birds are infected with other respiratory pathogens or in combination with environmental stressors. Virulent NDV isolates, the cause of ND—called exotic Newcastle disease (END) in the United States (U.S.), are not endemic in the U.S. and can spread rapidly leading to high mortality rates [9]. Symptoms of a virulent NDV infection in susceptible birds may include depression, respiratory distress, hemorrhage in multiple organs, neurological signs and acute death. ND vaccines are widely administered to reduce clinical disease from endemic infections with low virulence strains and can provide protection against disease but not infection with virulent outbreak viruses. Consequently, the primary control measure in the U.S. if an ND outbreak occurs is depopulation of infected or likely exposed animals. This can create a significant financial burden, for example the estimated cost for controlling the California 2002–2003 outbreak exceeded \$200 million [10].

In the U.S., and in many countries worldwide, ND prevention is focused on bio-security and the vaccination of poultry with both live and inactivated ND vaccines. Ideally vaccines are administered after maternal antibodies have waned which allows the induction of a good immunological response before the birds are likely to be exposed to a virulent strain of NDV, but because of differences in flock immunity, vaccination is rarely ideally implemented. Both live and inactivated vaccines have their advantages and disadvantages, which have been reviewed previously [11]. Today the strains of NDV used to produce ND vaccines in the U.S., such as LaSota and B1, are phylogenetically in the same genotype as viruses isolated in the 1940s, but are phylogenetically divergent from strains causing the recent outbreaks of ND in North America since the 1970s, such as Fontana/1972, Turkey North Dakota/1992, and California/2002 (see Fig. 1). It is widely recognized that because ND isolates are of one serotype, ND vaccines prepared with any ND lineage, given correctly, can protect poultry from clinical disease and mortality from a virulent ND virus challenge [12–14]. However, even as far back as 1953 the feasibility of one NDV vaccine being able to protect birds from ND without evaluating the factors for each individual outbreak has been questioned [15]. In 1972, Spalatin and Hanson noted that the new forms of NDV being isolated in the U.S. are able to infect vaccinated chickens and that these new viruses seem partially resistant to the antibodies induced by the current vaccines [16]. More recently, Kapczynski and King showed that current vaccination programs in commercial broilers in the U.S. are not completely effective at preventing clinical disease and virus shedding after experimental challenge with a recent virulent strain [10]. These results along with the susceptibility of vaccinated commercial layers to virulent NDV infection in the California 2002 outbreak suggests the current vaccination programs may not be optimized. The objective of this study was to compare the protection induced by ND vaccines prepared with viruses of five different NDV genotypes by assessing viral shed from vaccinates in addition to the standard observation of morbidity and mortality after challenge. The comparison was done with inactivated vaccines, the only feasible option to utilize the virulent CA 2002 NDV as both a vaccine antigen and a challenge virus. We found that vaccinating with a NDV homologous with the ND challenge virus induced high hemagglutination-inhibiting antibody titers and significantly reduced the amount of virus shed in oral secretions compared to the heterologous vaccines. Vaccines with the ability to reduce viral shed would enhance the role of vaccination in ND control.

2. Materials and methods

2.1. Eggs and chickens

Four-week-old, SPF White Leghorn (WL), chickens obtained from the Southeast Poultry Research Laboratory (SEPRL) flocks were separated into six vaccination groups of 16 birds each. The chickens were wing banded and kept in Horsfall isolation units in BSL 3 Ag facilities and allowed to acclimate for 2 days prior to their being vaccinated. Additional birds from this group were bled and tested by hemagglutination inhibition (HI) assay and ELISA (IDEXX, Westbrook, ME) to confirm that the flock was negative for NDV antibodies. Birds were given food and water ad libitum throughout the experiment. The SEPRL SPF WL flock was the source of the embryonated chicken eggs (ECE) utilized for virus isolation (VI), virus titrations and for the normal allantoic fluid for preparing the control vaccine and for diluting antigens after inactivation. The SEPRL Institutional Animal Care and Use Committee approved all animal experiments.

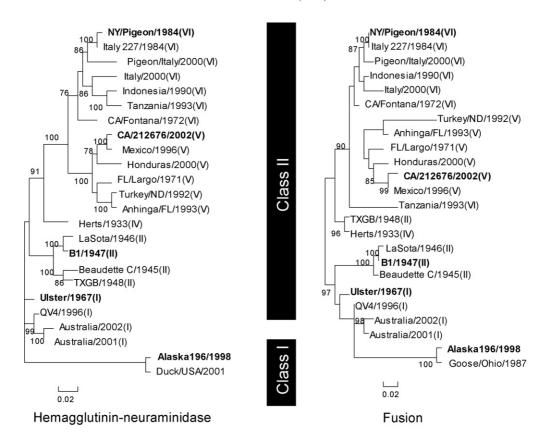


Fig. 1. Phylogenetic comparison of the full-length hemagglutinin-neuraminidase (HN) and fusion (F) proteins of representatives of the NDV classes and genotypes [7]. Viruses utilized to prepare vaccine antigens are in bold. Bootstrap values greater than 75 are noted. Ruler distance of 0.02 represents 2 amino acid changes per 100 amino acids.

2.2. Viruses and antigen preparation

We chose phylogenetically diverse ND viruses to use as vaccines: Ulster/1967 [2], B1/1947 [2], Pigeon/1984 (Pigeon84) [17], Alaska196/1998 [18] and California/212676/2002 (CA02) [19] (see Fig. 1 and Table 1). Ulster, a Class II Genotype I virus, was originally isolated in Northern Ireland and is used as a vaccine virus in

Table 1 Characterization of the ND viral strains used for the preparation of vaccines

| Antigen | HA titer ^a | | EID ₅₀ ^b | Class/genotype ^c |
|----------|-----------------------|-------------------|--------------------------------|-----------------------------|
| | Pre | Post | | |
| B1 | 512 | 384 | 109.7 | II/II |
| Ulster | 2048 | 2048 | $10^{9.3}$ | II/I |
| Pigeon84 | 32 | 1024 ^d | $10^{6.9}$ | II/VIb |
| AK196 | 2048 | 1024 | $10^{9.1}$ | I/NA ^e |
| CA02 | 512 | 512 | $10^{8.3}$ | II/V |

^a The HA titer of each antigen is listed pre- and post-inactivation with BPL.

that country. B1, a Class II Genotype II virus, is used worldwide as a live vaccine virus. Pigeon84, a Class II, Genotype VIb virus, is representative of the virulent pigeon paramyxoviruses and has been characterized previously as a mesogen in chickens [17,20]. Alaska196 is a Class I virus that was isolated in 1998 from a Northern Pintail and represents a group of viruses that are commonly found in waterfowl. Typically Class I isolates do not cause disease in poultry and genetically are highly divergent from the other isolates in the Genotypes of Class II [5]. There has been one velogenic Class I virus reported [21]. The CA02 virus, a Class II, Genotype V virus, is a velogen that is representative of the recent outbreak in the Southwestern U.S. and is used as a vaccine and challenge virus. Stocks of NDV were obtained from the SEPRL repository, and grown in 9-11 day-old SPF ECE by chorioallantoic sac inoculation. Pools of infective allantoic fluid were clarified via centrifugation at $1000 \times g$ for 15 min. Infectivity titers of the pools were determined by titration in ECE prior to inactivation, and hemagglutination (HA) titers were determined before and after inactivation (see Table 1) [22]. Allantoic fluid for each virus was inactivated with 0.1% beta-propiolactone (BPL) (Sigma, St. Louis, MO) [23] for 4 h at room temperature and kept overnight at 4 °C for hydrolysis of the BPL. Complete virus inactivation was confirmed by failure to recover virus after embryo inoculation [24]. Prior to being stored at

 $[^]b$ Embryo infectious dose 50 (EID $_{50}$) prior to inactivation is listed per 0.1 ml: all vaccines were adjusted to the equivalent EID $_{50}$ $10^{8.3}$ prior to inactivation.

^c Class and genotype [7].

^d Pigeon84 HA titer post-concentration.

^e Not applicable; no genotypes have been reported in Class I.

-70 °C, the pH of the pools of virus antigen as allantoic fluid was adjusted to 7.0 by adding sterile sodium bicarbonate (Gibco, Invitrogen Corporation, Grand Island, NY) [25].

2.3. Vaccine generation

Water-in-oil emulsion vaccines were prepared with virus antigen concentration the equivalent of $10^{8.3}$ EID₅₀ (median embryo infectious dose) of virus prior to BPL inactivation. To achieve this concentration B1, Ulster, and AK196 were diluted with normal allantoic fluid. Pigeon 84, having a lower EID₅₀ titer and HA titer, was concentrated by ultracentrifugation at $120,000 \times g$. CA02 was kept at the original concentration. Table 1 characterizes each of the viruses used for the vaccine preparation. The oil phase of the vaccine was made by adding 36 parts of Drakeol 6VR (Butler, PA), 3 parts of Span 80 (Sigma, St. Louis, MO) and 1 part of Tween 80 (Sigma, St. Louis, MO) for each vaccine to be made into a working solution. The oil phase was added to each of the virus antigens or normal allantoic fluid (the aqueous phase) to achieve a 4:1 ratio of oil to water as previously described [26]. Vaccines were prepared by homogenization in a Waring blender (Fisher Scientific International Inc., Hampton, NH) [27] 3 days prior to administration and kept at 4 °C prior to use.

2.4. Vaccination studies

Groups were subcutaneously vaccinated with 0.5 ml of their appropriate vaccines. Twenty-one days post-vaccination serum was collected and the birds were challenged with 10^{5.7} EID₅₀ of CA02 virus administered in 50 μl into the right eye and 50 µl into the choana. Oropharyngeal and cloacal swabs were collected on days 2, 4, 7 and 9 into 1.5 ml of brain heart infusion (BHI) broth (BD Biosciences, Sparks, MD) with a final concentration of gentamicin (200 μg/ml), penicillin G (2000 units/ml), and amphotericin B (4 μg/ml). Birds were monitored daily for clinical signs and death through day 14 post-challenge when they were bled and euthanized. Moribund chickens were euthanized with intravenous sodium pentobarbital at a dose of 100 mg/kg and counted dead for the next day. Necropsies were completed on selected birds post-challenge to assess the presence of gross pathological lesions.

2.5. VI, HA assay, HI assay, ELISA, monoclonal antibodies

Virus isolation (VI) and hemagglutination (HA) assays to identify virus positive fluids were conducted as described [19]. VI positive samples were titrated in SPF ECE [24]. All virus titers were calculated using the Spearman–Kärber method. Hemagglutination-inhibition (HI) assays (microbeta) were completed on pre- and post-challenge sera by testing all samples against their homologous and heterologous vaccine antigens [28]. ELISA assays (IDEXX,

Westbrook, ME) were also completed on the pre- and post-challenge sera according to the manufacturer's recommendations. Geometric mean titers (GMT) of HI antibodies were determined for each vaccination group. Each of the vaccine antigens were tested against NDV specific monoclonal antibodies (MAbs) to show antigenic variation among the NDV strains as described [29]. The National Veterinary Services Laboratory provided B79, 15C4, 10D11, AVS, and 161/167. P3A11, P11C9, P15D7, and P10B8, prepared at Southeast Poultry Research Laboratory, have been previously described [29]. Four HA units of each of the viral antigens were used in completing the HI assay of MAbs, and HI titers equal or greater than 16 are considered positive [17,30].

2.6. Nucleotide sequencing

All sequencing reactions were performed as previously described [31]. Pigeon84 HN and F, CA02 HN and F, and Alaska196 HN were sequenced from cDNA amplified by RT-PCR from Trizol LS (Invitrogen, Carlsbad, CA) extracted RNA using gene specific primers that are available upon request. Sequences have been deposited in GenBank® under the following accession numbers: EF520717 (CA02 HN), EF520718 (CA02 F), EF20715 (pigeon 84 HN), EF520716 (pigeon 84 F), EF520714 (AK196 HN), and EF612277 (AK196 F). Nucleotide sequences for the complete HN and F proteins for B1 (HN: AF309418 F: M24695) and Ulster (HN: M19478 F: M24694) are available from GenBank®.

2.7. Genetic analysis

The amino acid sequences of the HN and F proteins of the vaccine viruses used in the study were compared by phylogenetic analysis and pair-wise alignment of each isolate with CA 02 with the program Megalign (DNASTAR, Madison, WI). The sequences were aligned using the Lipman–Pearson Method with a Gonnet250 Protein weight matrix and amino acid similarities are shown in Table 2 [32].

Table 2
Deduced hemagglutinin-neuraminidase (HN) and fusion (F) protein similarity between the vaccine strain and the challenge NDV strain of CA02^a

| Vaccine | Amino acid similarity with challenge virus (%) | | | |
|-----------|--|-------|--|--|
| | HN | F | | |
| CA02 | 100.0 | 100.0 | | |
| Pigeon84 | 92.3 | 92.9 | | |
| Ulster | 90.7 | 89.7 | | |
| B1 | 89.3 | 88.1 | | |
| Alaska196 | 84.2 | 85.2 | | |

^a Amino acid similarity analysis and pair-wise alignment of each isolate with CA 02 were performed with the program Megalign (DNASTAR, Madison, WI): The amino acid sequences were aligned using the Lipman–Pearson method.

2.8. Phylogenetic tree assembly

Phylogenetic trees were constructed using the maximum likelihood method as implemented in the software package Phyml with the following parameters [33]: 100 boot-strapped data set, JTT model of amino acid substitution, fixed proportion of invariable sites, 4 substitution rate categories, 2 gamma distribution parameters and optimization of tree topology, branch lengths and rate parameters. Bootstrap values greater than 75 are reported.

2.9. Statistical analysis

Animal experiments were done with 16 chickens per treatment group with the exception of the B1 group in which one bird died pre-vaccination. Serology data are presented as geometric mean titers plus or minus (\pm) standard error. Group means were analyzed by ANOVA with Tukey's post hoc test when indicated. Significance is reported at the level of $P \le 0.05$.

3. Results

The five viruses chosen to be used as vaccines differed phylogenetically (Fig. 1, Table 1) and antigenically (Table 2). In evaluating the deduced similarity for the HN and F proteins between the CA02 challenge strain and the vaccine strains, Pigeon84 and Alaska196 are respectively the most and least genetically similar (Table 2). When using a panel of nine different monoclonal antibodies, each virus had a different antigenic pattern of reactivity compared to the CA02 virus antigenic pattern (data not shown). The CA02 virus shared six epitopes with Ulster and B1, but only two with Pigeon84 and Alaska196.

The chickens were vaccinated at 4 weeks of age and the pre-challenge serum 3 weeks after vaccination was analyzed with both a cross-HI assay and a commercial NDV ELISA test (Table 3). Up to fivefold titer differences were observed between Alaska196 and Pigeon84 antigens on mean HI titers when Alaska196 was the vaccine and a threefold difference

Table 3
Pre-challenge serology completed by micro-beta HI^a and ELISA (IDEXX)

| Vaccine | HI antigens | | | | | ELISA antigen | |
|----------|------------------|--------|----------|-------|------|-------------------|--|
| | B1 | Ulster | Pigeon84 | AK196 | CA02 | | |
| B1 | 291 ^b | 133 | 30 | 40 | 96 | 3676 ^c | |
| Ulster | 612 | 586 | 146 | 306 | 411 | 3045 | |
| Pigeon84 | 348 | 281 | 562 | 182 | 190 | 2816 | |
| AK196 | 334 | 174 | 146 | 829 | 198 | 3269 | |
| CA02 | 761 | 538 | 485 | 463 | 794 | 3292 | |

^a HI assays were completed with four HA units of each vaccine antigen to test pre-challenge serum of each vaccine group and group geometric mean titers are presented.

when Pigeon84 was the vaccine. There was a threefold difference in mean HI titers of the B1 vaccinates when tested with the B1 and CA02 antigen. The CA02 vaccine strain produced higher serum HI titers to homologous antigen as compared to the other vaccine strains. The ELISA titers from the B1 group were higher than all the other groups, although the B1 HI titers were the lowest. Post-challenge HI and ELISA titers, measured at 14 days, revealed an anamnestic response in all groups as expected since all vaccinates became infected with the challenge virus (data not shown).

The birds were challenged with the virulent CA02 strain and evaluated daily for morbidity and mortality. The control vaccinated birds and one bird from the Alaska196 vaccination group displayed conjunctivitis with severe depression, before dying or being euthanized between 4 and 6 days post-challenge. Necropsy of these controls and the Alaska196 bird revealed gross lesions consistent with a virulent NDV infection including petechial hemorrhages and edema in the conjunctiva of the lower eyelid, petechial hemorrhages in the thymus, and multifocal hemorrhages of the proventriculus and cecal tonsils. Hemorrhage of the tracheal mucosa posterior to the glottis, a unique lesion described consistently with this CA02 viral infection, was also observed [19].

Neurological signs were seen in two of the vaccinated birds: one B1 vaccinate and one CA02 vaccinate. The B1 vaccinate displayed torticollis, an inability to stand and slight body tremors. Upon necropsy at the end of the experiment, this bird was grossly normal except for petechial hemorrhages in the thymus. The CA02 vaccinate displayed a paralyzed wing, an inability to stand and to keep its head up. This bird displayed no gross lesions of a virulent NDV infection upon necropsy at the end of the experiment. Neither bird had the tracheal lesion previously described. The CA02 vaccinate with neurological lesions had pre-challenge serum HI antibodies to the CA02 antigen of 20 versus the mean titer of 1015 for the other 15 vaccinates in this group. The B1 bird with neurological signs had a HI antibody titer of 80 to the CA02 antigen, which was similar to the mean HI antibody titer of 118 for the other vaccinates in the B1 group.

All of the oral swabs from the control and vaccinated birds were positive on days 2 and 4 post-challenge with titers from all the groups peaking on day 4. By days 7 and 9 the number of vaccinated birds shedding virus was reduced. Table 4 demonstrates that there was no significant difference in the frequency of the number of birds shedding among the vaccination groups except for the number of positive cloacal swabs on day 2 for the Pigeon84 vaccinates. At 2 days postchallenge, vaccination with B1, Ulster, and Alaska196 had no effect on oral shedding of virus compared to controls (Fig. 2B) as measured by viral titers. However, both Pigeon 84 and CA02 caused a significant reduction in shedding compared to the controls. On day 4 the oral virus titers of the CA02 vaccinates were significantly reduced compared to the titers of the other vaccine strains as well as the controls (Fig. 2A and C). The heterologous NDV vaccine strains significantly reduced oral viral shed on day 4 (Fig. 2C) compared to the

^b Homologous responses are noted in bold.

^c ELISA group geometric mean titers are presented in the right column.

Table 4
Frequency of isolation of challenge virus in different vaccine groups

| Vaccine group | Days post-challenge samples collected | | | | | | | | |
|--------------------|---------------------------------------|--------|-------|-------|-----------------|-------|-------|-------|--|
| | 2 | | 4 | | 7 | | 9 | | |
| | $\overline{\mathrm{O^a}}$ | C_p | O | C | 0 | C | O | С | |
| Control | 16/16 ^c | 16/16 | 16/16 | 13/13 | NS ^d | NS | NS | NS | |
| B1 ^e | 15/15 | 08/15 | 15/15 | 06/15 | 05/15 | 05/15 | 02/15 | 00/15 | |
| Ulster | 16/16 | 10/16 | 16/16 | 07/16 | 02/16 | 00/16 | 00/16 | 03/16 | |
| Pigeon84 | 16/16 | 04/16* | 16/16 | 05/16 | 01/16 | 03/16 | 00/16 | 03/16 | |
| AK196 ^f | 16/16 | 12/16 | 16/16 | 07/16 | 00/15 | 02/15 | 00/15 | 02/15 | |
| CA02 | 16/16 | 10/16 | 16/16 | 04/16 | 02/16 | 02/16 | 02/16 | 00/16 | |

- ^a Oropharyngeal swabs.
- b Cloacal swabs.
- ^c Data are expressed as positive isolations/total number of swabs with one per bird.
- d No survivors
- ^e One bird from the B1 group died pre-vaccination.
- f One bird from the AK196 group died on day 5 post-challenge.
- * Significance from corresponding control, P<0.05.</p>

control vaccine, but that reduction was not as great as in the CA02 vaccinates. All of the cloacal swabs from the control group were positive on days 2 and 4. The cloacal swabs from the ND vaccinated groups contained low amounts of virus throughout the sampling period (Fig. 3A) and the number of birds shedding virus was decreased on days 2 and 4 post-infection compared to the control birds. Unlike the oral swabs, there was no significant difference in the virus titers from the cloacal swabs of those vaccinated groups except that Pigeon84 had significantly less virus isolated on day 2 compared with the amount isolated from Alaska196 (Fig. 3B). This difference disappeared by day 4 (Fig. 3C).

4. Discussion

The goal of this study was to determine if the antigenic distance of the vaccine strain, as described by phylogeny, can influence the amount of virus shed after infection with a virulent strain of NDV and thus impact decisions on vaccine formulation and challenge virus for potency testing. We identified four NDV isolates that represented four genotypes different from the CA02 outbreak strain to use in this study as vaccines that have different degrees of amino acid similarity to the CA02 HN and F proteins (Table 2). As shown in Fig. 1, these isolates represent the diversity found in both the HN and F proteins. Specific antibodies to both of these glycoproteins are known to be involved in the neutralization of NDV [34-41]. The majority of virulent ND strains isolated in North America since 1970 from poultry, psittacines and wild birds like cormorants and anhingas have been Class II Genotype V viruses that show nucleotide similarities to the Mexican isolates of 1996 and 1998 [42]. If there were to be another outbreak in the U.S., the etiological agent would likely be a virulent virus similar to the Class II Genotype V viruses of the recent past and not virulent viruses of the Class II Genotype II isolates like Texas GB that have not been isolated in the U.S. since the early 1970s.

In this study, the NDV vaccine homologous to the Mexican-like Class II Genotype V challenge virus (CA02) induced the highest titers of hemagglutination-inhibition antibodies using the CA02 virus as antigen when compared to the amounts induced by heterologous vaccines (Table 3). Most importantly, improved protection of vaccinated birds as measured by a significant decrease in challenge virus shedding in oropharyngeal swabs was also seen in the group vaccinated with the homologous vaccine (Fig. 2C). The HI assay detects antibodies to the HN surface antigen, which are known to correspond to antibodies that provide protection from disease. Each vaccine group gave the highest HI titers when the antigen used in the assay was homologous to the vaccine antigen, except for B1, which has been previously shown to respond poorly in this regard [43]. The cross HI titers in Table 3 also show that the HI titers can vary greatly depending on the antigen used for testing. For example the B1 vaccinated birds had a GMT HI titer of 291 when compared with B1 antigen, but a titer of 96 when using CA02, the challenge strain as antigen. Using the vaccine antigen and not the probable challenge antigen in evaluating the GMT HI response could lead to an over estimation of the immune response and the potential level of protection they induced (Table 3). Testing these same vaccinates against the antigen of the likely challenge virus will give a better indication of the type of protection these birds will have. We also found that the ELISA titers (Table 3) for the B1 vaccinates had the highest NDV antibody response even though the B1 vaccinates had the lowest HI titers to the CA02 challenge antigen. These results suggest that either the ELISA antigen had greater homology with the B1 virus or it simply reflects the differences in levels of antibodies to conserved structural proteins other than the HN in the response measured by ELISA. The similarity of ELISA antibody titers among all vaccine groups in contrast to the variability in HI titers indicates the lesser role of the HN in the induction of the antibodies assayed by ELISA. Consequently, the ELISA response may not be as useful

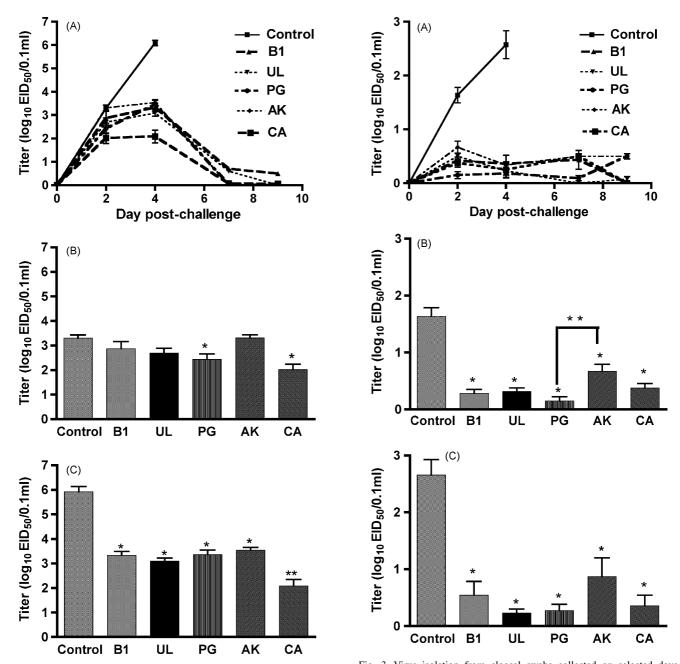


Fig. 2. Virus isolation from oropharyngeal (oral) swabs collected on selected days after CA02 END virus challenge of all treatment groups at 21 days post-vaccination. (A) Mean virus titers of oral swabs of all groups on all sample days. All control animals were dead by day 6. (B) Comparison of oral virus titers at 2 day post-challenge: asterisk (*) indicates significant difference from Control and AK. (C) Comparison of oral virus titers at 4 day post-challenge: asterisk (*) indicates significant difference between control, double asterisk (**) indicate significant difference between control and all other treatments. Data (mean ± S.E.) were analyzed by ANOVA followed by Tukey's multiple comparison test. B1: B1, UL: Ulster, PG: Pigeon84, AK: AK196, CA: CA02.

as the HI in predicting the level of protection induced by vaccination.

Although virus shed has not been widely reported as a method of monitoring protection induced by ND vaccines

Fig. 3. Virus isolation from cloacal swabs collected on selected days after CA02 END virus challenge of all treatment groups at 21 days post-vaccination. (A) Mean virus titers of cloacal swabs of all groups on all sample days. All control animals were dead by day 6. (B) Comparison of cloacal virus titers at 2 day post-challenge: asterisk (*) indicates significant difference from control; double asterisk (**) indicate significance difference between Alaska and Pigeon. (C) Comparison of cloacal virus titers at 4 day post-challenge: asterisk (*) indicates significant difference from control. Data were analyzed by ANOVA followed by Tukey's multiple comparison test. B1: B1, UL: Ulster, PG: Pigeon84, AK: AK196, CA: CA02.

[10], there are many reports of avian influenza (AI) vaccines being able to reduce the number of vaccinated birds shedding challenge virus [44,45] and also of them being able to reduce the amount of challenge virus shed from the respiratory tracts [45–47]. Notably, one report describes a similar pattern seen in these NDV experiments with significant reductions in

oropharyngeal shedding with a homologous vaccine and no differences in cloacal shedding between vaccine groups [48]. While antigenic drift does appear to be happening with NDV isolates throughout the world, it is occurring at a much slower scale than that seen with AI viruses [5,7,49–51]. In addition to shedding less virus into the environment, birds vaccinated for avian influenza have been shown to be more resistant to challenge by requiring a larger amount of virus to become infected [52].

Control of Newcastle disease primarily consists of vaccination of flocks and culling of infected or likely infected birds. Current vaccine strategies can be effective in controlling serious illness and death in infected birds, but do not prevent infection and shedding of virus. In the U.S. where virulent ND viruses are not endemic, vaccination programs are not intensive to minimize post-vaccinal reaction [11]. Transmission of virus even in a well-vaccinated flock can occur because some of the birds will have had a poor vaccine response and will be susceptible to infection. This was seen in layer flocks during the CA02 outbreak. However, in broilers, because of their short life spans and the need to balance immune response with vaccine reactions, this group often has an immune response that does not provide complete clinical protection and allows high levels of virus shedding on challenge [10]. In countries where a virulent challenge is likely, the vaccination programs may be more intensive and consequently transmission of a virulent virus may be reduced. The goal of the current study was to determine if it is possible to reduce viral shedding, and presumably, the spreading of the virus and the consequent disease, through an improved vaccine strategy. The current vaccines used to prevent ND were derived from strains isolated decades ago. In the last 50 years there has been a major shift in the types of strains of NDV that have been identified as circulating in poultry, although they still remain as a single serotype. The viral strains of greatest concern today exhibit considerable antigenic and sequence variation from the original vaccine strains (Table 2 and Fig. 1). We hypothesized that if birds were vaccinated with viruses that were more antigenically similar to the challenge strain that they would shed reduced amounts of challenge virus. Indeed, the data from this study support this hypothesis.

Historically, protection induced by NDV vaccines is tested from a challenge with Texas GB/1948 in the U.S., a Class II, Genotype II virus and with Herts/1933 in Europe, a Class II, Genotype IV virus. These challenge strains do not represent the virus lineages that are currently seen in North America and around the world. Currently, protection from NDV, as evaluated for biological regulatory purposes, is defined as protection induced by vaccines against morbidity and mortality after challenge. With this definition and based on these data, the lineage of the challenge strain used to test vaccines will likely not make a difference. However in this study vaccines formulated to be similar to the challenge virus induced better protection in vaccinates as measured by the reduction in the shedding of virus after a virulent challenge. Thus, by

formulating ND vaccines with a virus similar to the most likely outbreak virus it may be feasible to induce an immune response that not only protects against morbidity and mortality, but also against dissemination of the virulent virus.

Acknowledgments

The authors would like to thank Tim Olivier, Suzanne DeBlois, and Dawn Williams-Coplin for their excellent technical assistance, and Roger Brock for animal care assistance. We extend our appreciation to Dr. Mia Kim for her assistance with the animal studies. USDA, ARS CRIS project 6612-32000-049, supported this research.

References

- [1] Mayo MA. A summary of taxonomic changes recently approved by ICTV. Arch Virol 2002;147(8):1655–63.
- [2] Alexander DJ. Newcastle disease, other avian paramyxoviruses, and pneumovirus infections. In: Saif JM, Barnes HJ, Glisson JR, Fadly AM, McDougald LR, Swayne DE, editors. Diseases of poultry. 11th ed. Ames: Iowa State University Press; 2003. p. 63–99.
- [3] Seal BS, King DJ, Sellers HS. The avian response to Newcastle disease virus. Dev Comp Immunol 2000;24(2–3):257–68.
- [4] Alexander DJ, Morris HT, Pollitt WJ, Sharpe CE, Eckford RL, Sainsbury RM, et al. Newcastle disease outbreaks in domestic fowl and turkeys in Great Britain during 1997. Vet Rec 1998;143(8):209–12.
- [5] Aldous EW, Mynn JK, Banks J, Alexander DJ. A molecular epidemiological study of avian paramyxovirus type 1 (Newcastle disease virus) isolates by phylogenetic analysis of a partial nucleotide sequence of the fusion protein gene. Avian Pathol 2003;32(3):239–56.
- [6] Ballagi-Pordany A, Wehmann E, Herczeg J, Belak S, Lomniczi B. Identification and grouping of Newcastle disease virus strains by restriction site analysis of a region from the F gene. Arch Virol 1996;141(2):243–61.
- [7] Czegledi A, Ujvari D, Somogyi E, Wehmann E, Werner O, Lomniczi B. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res 2006;120:36–48.
- [8] Hanson RP, Crook E, Brandly CA. Comparisons of immunogenicity of five strains of Newcastle disease virus as formalinized vaccines. Vet Med 1951;46:451–2.
- [9] USDA. The Office of the Federal Register National Archives and Records Administration Code of Federal Regulations. Title 9 Animals and Animal Products: Chapter I APHIS, Department of Agriculture; Part 94; 2006, p. 490.
- [10] Kapczynski DR, King DJ. Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. Vaccine 2005;23(26):3424–33.
- [11] Senne DA, King DJ, Kapczynski DR. Control of Newcastle disease by vaccination. Dev Biol (Basel) 2004;119:165–70.
- [12] Butterfield WK, Dardiri AH, Yedloutschnig RJ. Protection of chickens afforded by commercial lentogenic vaccines against challenge exposure to velogenic Newcastle disease virus. Avian Dis 1973;17(2):279–82.
- [13] Benson HN, Wenger DR, Beard PD. Efficacy of a commercial Newcastle vaccine against velogenic viscerotropic Newcastle disease virus. Avian Dis 1975;19(3):566–72.
- [14] Beard CW, Villegas P, Glisson JR. Comparative efficacy of the B-1 and VG/GA vaccine strains against velogenic viscerotropic Newcastle disease virus in chickens. Avian Dis 1993;37(1):222–5.

- [15] Upton E, Hanson RP, Brandly CA. Antigenic differences among strains of Newcastle disease virus. Proc Soc Exp Biol Med 1953;84(3): 691–3
- [16] Spalatin J, Hanson RP. Comparison of B1 and Ulster strains of Newcastle disease virus as vaccines against the viscerotropic form of disease. In: Proceedings of the 21st Western poultry disease conference and 6th poultry health symposium. 1972. p. 66–9.
- [17] King DJ. Avian paramyxovirus type 1 from pigeons: isolate characterization and pathogenicity after chicken or embryo passage of selected isolates. Avian Dis 1996;40(3):707–14.
- [18] Kim LM, King DJ, Suarez DL, Wong CW, Afonso CL. Characterization of Class I Newcastle disease virus isolates from Hong Kong bird markets and detection using real-time reverse transcription PCR. J Clin Microbiol 2007;45(4):1310–4.
- [19] Wakamatsu N, King DJ, Kapczynski DR, Seal BS, Brown CC. Experimental pathogenesis for chickens, turkeys, and pigeons of exotic Newcastle disease virus from an outbreak in California during 2002–2003. Vet Pathol 2006;43(6):925–33.
- [20] Kommers GD, King DJ, Seal BS, Brown CC. Virulence of pigeonorigin Newcastle disease virus isolates for domestic chickens. Avian Dis 2001;45(4):906–21.
- [21] Alexander DJ, Campbell G, Manvell RJ, Collins MS, Parsons G, McNulty MS. Characterisation of an antigenically unusual virus responsible for two outbreaks of Newcastle disease in the Republic of Ireland in 1990. Vet Rec 1992;130(4):65–8.
- [22] Thayer SG, Beard CW. Serological procedures. In: Swayne DE, editor. A laboratory manual for the isolation and identification of avian pathogens. 4th ed. Kennett Square: American Association of Avian Pathologists; 1998. p. 255–66.
- [23] Spradbrow PB, Samuel JL. Oral Newcastle disease vaccination with V4 virus in chickens: comparison with other routes. Aust Vet J 1991;68(3):114–5.
- [24] Alexander DJ. Newcastle disease virus and other avian paramyxoviruses. In: Swayne DE, Glisson JR, Jackwood MW, Pearson JE, Reed WM, editors. A laboratory manual for the isolation and identification of avian pathogens. 4th ed. Kennett Square: American Association of Avian Pathologists; 1998. p. 156–63.
- [25] Budowsky EI, Smirnov YuA, Shenderovich SF. Principles of selective inactivation of viral genome. VIII. The influence of beta-propiolactone on immunogenic and protective activities of influenza virus. Vaccine 1993;11(3):343–8.
- [26] Stone HD, Brugh M, Beard CW. Influence of formulation on the efficacy of experimental oil-emulsion Newcastle disease vaccines. Avian Dis 1983:27:688–97.
- [27] Stone HD, Brugh M, Hopkins SR, Yoder HW, Beard CW. Preparation of inactivated oil-emulsion vaccines with avian viral or Mycoplasma antigens. Avian Dis 1978;22(4):666–74.
- [28] King DJ. Influence of chicken breed on pathogenicity evaluation of velogenic neurotropic Newcastle disease virus isolates from cormorants and turkeys. Avian Dis 1996;40(1):210–7.
- [29] Kommers GD, King DJ, Seal BS, Brown CC. Pathogenesis of chickenpassaged Newcastle disease viruses isolated from chickens and wild and exotic birds. Avian Dis 2003;47(2):319–29.
- [30] International Office of Epizootics. Biological Standards Commission. Manual of diagnostic tests and vaccines for terrestrial animals: mammals, birds and bees. 5th ed. Paris: Office international des âepizooties; 2004.
- [31] Kim LM, Afonso CL, Suarez DL. Effect of probe-site mismatches on detection of virulent Newcastle disease viruses using a fusion-gene realtime reverse transcription polymerase chain reaction test. J Vet Diagn Invest 2006;18(6):519–28.
- [32] Lipman DJ, Pearson WR. Rapid and sensitive protein similarity searches. Science 1985;227(4693):1435–41.
- [33] Guindon S, Lethiec F, Duroux P, Gascuel O. PHYML Online-a web server for fast maximum likelihood-based phylogenetic inference. Nucleic Acids Res 2005;33(Web Server issue):W557–9.

- [34] Reynolds DL, Maraqa AD. Protective immunity against Newcastle disease: the role of antibodies specific to Newcastle disease virus polypeptides. Avian Dis 2000;44(1):138–44.
- [35] Merz DC, Scheid A, Choppin PW. Importance of antibodies to the fusion glycoprotein of paramyxoviruses in the prevention of spread of infection. J Exp Med 1980;151(2):275–88.
- [36] Boursnell ME, Green PF, Samson AC, Campbell JI, Deuter A, Peters RW, et al. A recombinant fowlpox virus expressing the hemagglutinin-neuraminidase gene of Newcastle disease virus (NDV) protects chickens against challenge by NDV. Virology 1990;178(1):297–300.
- [37] Boursnell ME, Green PF, Campbell JI, Deuter A, Peters RW, Tomley FM, et al. A fowlpox virus vaccine vector with insertion sites in the terminal repeats: demonstration of its efficacy using the fusion gene of Newcastle disease virus. Vet Microbiol 1990;23(1–4):305–16.
- [38] Kamiya N, Niikura M, Ono M, Kai C, Matsuura Y, Mikami T. Protective effect of individual glycoproteins of Newcastle disease virus expressed in insect cells: the fusion protein derived from an avirulent strain had lower protective efficacy. Virus Res 1994;32(3):373–9.
- [39] Loke CF, Omar AR, Raha AR, Yusoff K. Improved protection from velogenic Newcastle disease virus challenge following multiple immunizations with plasmid DNA encoding for F and HN genes. Vet Immunol Immunopathol 2005;106(3–4):259–67.
- [40] Park MS, Steel J, Garcia-Sastre A, Swayne D, Palese P. Engineered viral vaccine constructs with dual specificity: avian influenza and Newcastle disease. Proc Natl Acad Sci USA 2006;103(21):8203–8.
- [41] Umino Y, Kohama T, Kohase M, Sugiura A, Klenk HD, Rott R. Biological functions of monospecific antibodies to envelope glycoproteins of Newcastle disease virus. Arch Virol 1984;81(1–2):53–65.
- [42] Pedersen JC, Senne DA, Woolcock PR, Kinde H, King DJ, Wise MG, et al. Phylogenetic relationships among virulent Newcastle disease virus isolates from the 2002–2003 outbreak in California and other recent outbreaks in North America. J Clin Microbiol 2004;42(5):2329–34.
- [43] Kendal AP, Allan AW. Comparative studies of Newcastle disease viruses: virulence, antigenic specificity, and growth kinetics. Microbios 1970;2(7–8):273–84.
- [44] Taylor J, Weinberg R, Kawaoka Y, Webster RG, Paoletti E. Protective immunity against avian influenza induced by a fowlpox virus recombinant. Vaccine 1988;6(6):504–8.
- [45] Webster RG, Taylor J, Pearson J, Rivera E, Paoletti E. Immunity to Mexican H5N2 avian influenza viruses induced by a fowl pox-H5 recombinant. Avian Dis 1996;40(2):461–5.
- [46] Swayne DE, Beck JR, Mickle TR. Efficacy of recombinant fowl poxvirus vaccine in protecting chickens against a highly pathogenic Mexican-origin H5N2 avian influenza virus. Avian Dis 1997;41(4):910–22.
- [47] Swayne D, Beck JR, Garcia M, Stone HD. Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines. Avian Pathol 1999;28(3):245–56.
- [48] Swayne DE, Garcia M, Beck JR, Kinney N, Suarez DL. Protection against diverse highly pathogenic H5 avian influenza viruses in chickens immunized with a recombinant fowlpox vaccine containing an H5 avian influenza hemagglutinin gene insert. Vaccine 2000;18(11–12):1088–95.
- [49] Seal BS, King DJ, Meinersmann RJ. Molecular evolution of the Newcastle disease virus matrix protein gene and phylogenetic relationships among the paramyxoviridae. Virus Res 2000;66(1):1–11.
- [50] Toyoda T, Sakaguchi T, Hirota H, Gotoh B, Kuma K, Miyata T, et al. Newcastle disease virus evolution. II. Lack of gene recombination in generating virulent and avirulent strains. Virology 1989;169(2):273–82.
- [51] Lee CW, Senne DA, Suarez DL. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. J Virol 2004;78(15):8372–81.
- [52] Marangon S, Capua I. Control of avian influenza in Italy: from stamping out to emergency and prophylactic vaccination. Dev Biol (Basel) 2006;124:109–15.